Increased selective uptake *in vivo* and *in vitro* of oxidized cholesteryl esters from high-density lipoprotein by rat liver parenchymal cells

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Oxidation of low-density lipoprotein (LDL) leads initially to the formation of LDL-associated cholesteryl ester hydroperoxides (CEOOH). LDL-associated CEOOH can be transferred to high-density lipoprotein (HDL), and HDL-associated CEOOH are rapidly reduced to the corresponding hydroxides (CEOH) by an intrinsic peroxidase-like activity. We have now performed *in vivo* experiments to quantify the clearance rates and to identify the uptake sites of HDL-associated [^{3}H]Ch18:2-OH in rats. Upon injection into rats, HDL-associated [^{3}H]Ch18:2-OH is removed more rapidly from the circulation than HDL-associated [^{3}H]Ch18:2. Two minutes after administration of [^{3}H]Ch18:2-OH-HDL, 19.6±2.6% (S.E.M.; $n = 4$) of the label was taken up by the liver as compared with 2.4±0.25% (S.E.M.; $n = 4$) for [^{3}H]Ch18:2-HDL. Organ distribution studies indicated that only the liver and adrenals exhibited preferential uptake of [^{3}H]Ch18:2-OH as compared with [^{3}H]Ch18:2, with the liver as the major site of uptake. A cell-separation procedure, employed 10 min after injection of [^{3}H]Ch18:2-OH-HDL or [^{3}H]Ch18:2-HDL, demonstrated that within the liver only parenchymal cells take up HDL-CE by the selective uptake pathway. Selective uptake by parenchymal cells of [^{3}H]Ch18:2-OH was 3-fold higher than that of [^{3}H]Ch18:2, while Kupffer and endothelial cell uptake of the lipid tracers reflected HDL holoparticle uptake (as analysed with iodinated versus cholesteryl ester-labelled HDL). The efficient uptake of [^{3}H]Ch18:2-OH by parenchymal cells was coupled to a 3-fold increase in rate of radioactive bile acid secretion from [^{3}H]Ch18:2-OH-HDL as compared with [^{3}H]Ch18:2-HDL. *In vitro* studies with freshly isolated parenchymal cells showed that the association of [^{3}H]Ch18:2-OH-HDL at 37 °C exceeded [^{3}H]Ch18:2-HDL uptake almost 4-fold. Our results indicate that HDL-associated CEOH are efficiently and selectively removed from the blood circulation by the liver *in vivo*. The selective liver uptake is specifically exerted by parenchymal cells and coupled to a rapid biliary secretion pathway. The liver uptake and biliary secretion route may allow HDL to function as an efficient protection system against potentially atherogenic CEOOH.

INTRODUCTION

Oxidatively modified low-density lipoproteins (LDL) are suggested to play an important role in the development of atherosclerosis [1]. Oxidation of LDL can proceed via an oxidative chain reaction, whereby the formation of lipid hydroperoxides is followed by decomposition to reactive aldehydes, which in turn can modify amino acid residues of the apoprotein [2]. The earliest lipid hydroperoxides that are formed are mainly cholesteryl linoleate hydroperoxides (Ch18:2-OH) and phospholipid hydroperoxides [3]. Oxidatively modified LDL is recognized by specific scavenger receptors, allowing the unbridled accumulation of cellular lipids [4]. High-density lipoproteins (HDL) might exert anti-atherosclerotic effects through various mechanisms. HDL can accept excessive cholesterol from macrophages and other cell types as an initial step of the so-called reverse cholesterol transport pathway [5], reviewed in [6]. Excess cholesterol is taken up primarily by small HDL particles with pre-beta mobility [7], and after extensive conversion of the HDL particle, including esterification of free cholesterol by lecithin–cholesterol acyltransferase, transported to the liver for excretion in bile. Either HDL-cholesteryl ester (HDL-CE) can be transferred to LDL and very-low-density lipoproteins (VLDL) by the action of the cholesteryl ester transfer protein (CETP) or delivered directly to the liver. The direct uptake of HDL-CE by the liver parenchymal cells is not coupled to holoparticle uptake. Studies by Pittman and coworkers [8,9] indicated that CE are taken up selectively by liver parenchymal cells, exceeding uptake of the protein moiety of HDL. Within the liver, only parenchymal cells showed selective uptake, which is directly coupled to efficient biliary secretion of the bile acids formed [10].

A second mechanism for the anti-atherogenic action of HDL is related to its anti-oxidative properties [11]. Several mechanisms have been proposed by which HDL can prevent oxidative damage of LDL. First, HDL is suggested to trap oxidative substances, e.g. HDL is reported to chelate transition metals via particle-associated ceruloplasmin and transferrin, thus preventing LDL oxidation [12]. Also, an albumin-containing HDL subfraction prevented Cu^{2+}-catalysed oxidation of LDL by scavenging oxidation products [13]. Secondly, several HDL-associated enzymes may protect against oxidation of LDL (reviewed in [11]). Paraoxonase is the best known example of an HDL-associated protein which has been shown to prevent LDL oxidation by decreasing the formation of lipid hydroperoxides [14].

Recently, a third HDL-mediated anti-oxidative defense system has been suggested, involving the transfer of oxidized lipids from LDL to HDL [15,16]. Bowry et al. [17] showed that HDL is the predominant carrier of cholesteryl ester hydroperoxides (CEOOH) in humans. The first step in LDL oxidation does involve hydroperoxide formation, and it is suggested that HDL may accept CEOOH from LDL. It has been shown that CETP can mediate this transfer [18]. Moreover, a HDL-associated

Abbreviations used: CE, cholesteryl ester; CEOH, cholesteryl ester hydroxide; CEOOH, cholesteryl ester hydroperoxide; CETP, cholesteryl ester transfer protein; $Ch_{18:2}$-OOH, cholesteryl linoleate hydroperoxide; Ch18:2-OH, cholesteryl linoleate hydroxide; Ch18:2, cholesteryl linoleate; DMEM, Dulbecco's modified Eagle's medium; HDL, high-density lipoprotein; LCAT, lecithin cholesterol acyl transferase; LDL, low-density lipoprotein.

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hydroperoxide-reducing activity was found that reduces the reactive CEOOH to the less reactive cholesteryl ester hydroxides (CEOH) [16]. The question then arises about the subsequent fate of HDL-associated CEOH. *In vitro* studies have shown that both CEOOH and CEOH from HDL are taken up by HepG2 cells at a greater rate than native cholesteryl esters, while the uptake of the protein moiety of HDL was unchanged [15]. Our present goal was to analyse whether this selective HDL-mediated CEOH delivery occurs *in vivo*, which organs and/or cell types are involved, and to what extent the removal of HDL-CEOH from the blood circulation is coupled to biliary secretion.

EXPERIMENTAL

Materials

[Cholesteryl-1,2,6,7-^{3}H(*N*)] linoleate (Ch18:2) was purchased from DuPont NEN (Vienna, Austria). [1α,2α(n)-^{3}H]Cholesteryl linoleate ([^{3}H]CH18:2) and ^{125}I (carrier free) in NaOH were obtained from Amersham (Little Chalfont, Bucks., U.K.). 2,2′-Azobis-(2,4-dimethyl-valeronitrile) (AMVN) was purchased from Polyscience, (Warrington, PA, U.S.A.). Egg yolk phosphatidyl choline was obtained from Fluka (Buchs, Switzerland), and the PL phospholipids kit, the cholesterol oxidase–peroxidase aminophenazone (CHOD–PAP) kit and the glycerol phosphate oxidase–peroxidase aminophenazone (GPO–PAP) kit were from Boehringer Mannheim (Mannheim, Germany). Ethylmercurithiosalicylate (thimerosal), BSA (fraction V) and collagenase type I and type IV were from Sigma (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was from GIBCO (Irvine, Scotland). All other chemicals were of analytical grade.

Label preparation

[^{3}H]Cholesteryl linoleate hydroperoxide ([^{3}H]Ch18:2-OOH) was prepared by peroxidation of Ch18:2 with the lipid-soluble peroxyl radical generator AMVN. [^{3}H]Ch18:2 (200 μCi) in toluene (final volume 1 ml) was peroxidized with AMVN (37 °C, 0.4 M, 5 h). The incubation mixture was dried under nitrogen and purified by reverse-phase HPLC on an LC-18 column, as described in [19]. The fraction eluting at between 8 and 9 min (corresponding to the retention time of an unlabelled Ch18:2-OOH standard) was collected and dried in a SpeedVac (Savant, France). The resulting [^{3}H]Ch18:2-OOH was then chemically reduced to the corresponding [^{3}H]Ch18:2-OH by $NaBH_4$ (10 mg) reduction in methanol (1 ml) on ice (1 h) as described by Van Kuijk et al. [20]. [^{3}H]Ch18:2-OH was extracted into hexane and purified in a second HPLC step (LC-18 column; mobile phase acetonitrile/propan-1-ol/H_2O, 22:27:1 by vol.) [21].

Isolation and labelling of HDL

Human HDL was isolated from the blood of healthy volunteers by differential ultracentrifugation as described by Redgrave et al. [22]. HDL ($1.063 < d < 1.21$) was dialysed against PBS/1 mM EDTA and labelled with either [^{3}H]Ch18:2 or [^{3}H]Ch18:2-OH by exchange from donor particles, as reported previously [10]. Donor particles were formed by sonication of egg yolk phosphatidyl choline supplemented with 50 μCi of either [^{3}H]Ch18:2 or [^{3}H]Ch18:2-OH. Sonication was carried out with an MSE soniprep 150 for 40 min (amplitude 12 μm) at 52 °C under a constant stream of argon in a 0.1 M KCl/10 mM Tris/1 mM EDTA/0.025% NaN_3 buffer, pH 8.0. Donor particles with a density of 1.03 g/ml were isolated by density gradient ultracentrifugation. The phospholipid content of the particles was measured by an enzymic colorimetric assay.

HDL was labelled by incubating HDL with donor particles (mass ratio HDL-protein:particle phospholipid = 8:1) in the presence of human lipoprotein-deficient serum as the CETP source (1:1, v/v) for 8 h at 37 °C in a shaking water bath under argon. Ethylmercurithiosalicylate (thimerosal) (20 mM) was added to stimulate CE transfer and to inhibit phospholipid transfer and lecithin–cholesterol acyltransferase activity [23]. Radiolabelled HDL was then isolated by density gradient ultracentrifugation. The distribution of [^{3}H]Ch18:2 and [^{3}H]Ch18:2-OH over the HDL subfractions was studied by fractionation of a KBr gradient (1.063 g/ml–1.24 g/ml, 6 h in a Beckman TLX 120 benchtop centrifuge at 417000 *g* in 500 μl fractions. The distributions of [^{3}H]Ch18:2 and [^{3}H]Ch18:2-OH over the HDL density range were completely superimposable, indicating that both labels distribute similarly between HDL subfractions as separated by density ultracentrifugation. Subsequently, the labelled HDL was dialysed against PBS/1 mM EDTA and passed through a heparin–Sepharose affinity column to remove apo E-containing particles [24]. The HDL fraction was checked for the absence of apo E by SDS/10%-PAGE, followed by Coomassie Blue staining. After the labelling procedure the radiolabelled HDL was checked for hydrolysis of the cholesteryl ester labels by a Bligh and Dyer extraction [25] followed by TLC. The effect of the labelling procedure on HDL was analysed by measurement of phospholipid, cholesterol, CE and triglyceride content (with the PL kit, CHOD-PAP kit and GPO-PAP kit respectively). The density, electrophoretic α-mobility and particle size (photon correlation spectroscopy, System 4700 C, Malvern Instruments) were also analysed. Labelled HDL was only used when no change was observed in the measured composition or physical characteristics as compared with the original unlabelled HDL batch. Additionally, to exclude the possibility that endogenous HDL lipids or tracers were oxidized during the labelling procedure, HPLC analyses of lipid extracts obtained from [^{3}H]Ch18:2-labelled HDL were performed. HDL lipids were extracted into methanol/hexane (1:5), and the hexane phase, containing the neutral lipids, was dried and analysed by HPLC. Separation was performed on an LC-18 column (25 × 0.2 cm) with methanol/propan-2-ol (1:1) as the mobile phase (flow rate: 1 ml/min). Unoxidized lipids were detected at 210 nm while the 234 nm trace, in combination with radiometric detection (0–18.5 keV), was used to monitor the possibility of lipid peroxidation during the labelling procedure. Ch18:2-OH or Ch18:2-OOH did not appear in the lipid fraction of HDL, thus [^{3}H]Ch18:2 remained unoxidized throughout the entire labelling procedure. HDL was iodinated by the ICl method of McFarlane [26] as modified by Bilheimer et al. [27].

Serum decay, liver association and tissue distributions

Male Wistar WU rats (200–250 g) were anaesthetized by intraperitoneal injection of 20 mg of Nembutal. Body temperature was maintained by a heating lamp. After opening of the abdomen, radiolabelled HDL was injected into the inferior vena cava. At the indicated time points, blood sampling and liver lobule excision were performed as described previously [28]. After the last blood and liver samples had been taken, individual tissues were excised and weighed. Liver and other tissue samples were combusted in a Packard sample oxidizer 306 and counted for radioactivity in a Packard liquid-scintillation counter. Liver and other tissue-associated radioactivities were corrected for the radioactivity assumed to be present in the serum at the time of sampling, as determined by injection of ^{125}I-BSA (Th.J. C. Van Berkel and J. K. Kruijt, unpublished work).

Cell isolation at 4 °C

The hepatic cellular distribution of HDL was studied by using a low-temperature cell-isolation technique as described [28]. Rats were anaesthetized and injected with radiolabelled HDL. Ten minutes after injection, the vena porta was cannulated and the liver was perfused with oxygenated Hanks buffer, containing Hepes (1.6 g/l), pH 7.4 at 4 °C. After an 8 min perfusion a liver lobule was tied off for determination of total liver uptake. The perfusion was continued for 15 min with Hanks/Hepes buffer containing 0.05% (w/v) collagenase (type I; Sigma) and 1 mM $CaCl_2$. Parenchymal cells were isolated by mincing the liver in Hanks buffer containing 0.3% (w/v) BSA, filtering through nylon gauze and centrifuging for three 30 s periods at 50 *g*. The pellets consisted of pure parenchymal cells, as judged by light microscopy. The supernatants were centrifuged for 10 min at 400 *g* in order to harvest the non-parenchymal cells. The remainder on the nylon gauze was incubated with Hanks/Hepes/BSA buffer containing 0.25% Pronase for 15 minutes at 4 °C. This cell suspension was centrifuged for 10 min at 400 *g* and all non-parenchymal cell pellets were combined. By means of centrifugal elutriation the non-parenchymal cells were subdivided into an endothelial cell fraction and a Kupffer cell fraction [29]. The purity of each cell fraction was checked by light microscopy after staining for peroxidase activity, and samples were counted for radioactivity.

Bile sampling

Bile was collected from unrestrained male Wistar WU rats (400–450 g) as reported previously [30]. Access to chow and water was *ad libitum*. Rats were equipped with permanent catheters in the bile duct, duodenum and vena jugularis. The bile duct and duodenum catheters were connected after surgery and remained so during recovery of the rat (1 week). Lipoproteins were introduced via the catheter in the vena jugularis. Bile samples were collected in 1 h fractions during the indicated time, and counted for radioactivity. Bile acids were separated from cholesterol and CE by means of a Bligh and Dyer extraction [25]. Cholesterol and CE were separated by TLC. Spots were visualized by staining with $MnCl_2$ [31], scraped off and counted for radioactivity.

In vitro studies with freshly isolated rat hepatocytes

For *in vitro* studies, parenchymal liver cells were isolated by perfusion of the liver with collagenase at 37 °C as described [29]. The viability (> 95%) of the parenchymal cells obtained was checked by Trypan Blue exclusion. The cells were resuspended in DMEM supplemented with 2% (w/v) BSA, pH 7.4. Parenchymal cell protein (2–3 mg) was incubated with the indicated amounts of HDL for various periods in 1 ml of DMEM/2% BSA at 37 °C. After incubation the cells were washed twice in PBS/0.2% (w/v) BSA, pH 7.4, and subsequently washed in PBS without BSA. The cells were lysed in 0.1 M NaOH and the protein content and radioactivity were determined.

Protein determination

Protein was determined according to the method of Lowry et al. [32] with BSA as standard.

RESULTS

Serum decay and liver association

Upon injection of [^{3}H]Ch18:2-OH-labelled HDL into rats, a rapid removal of the label from blood is observed, markedly exceeding the rate of [^{3}H]Ch18:2 removal from HDL (~ 3-fold) (Figure 1). Analysis of the liver uptake indicates that within 2 min 19.6±2.6% (S.E.M.; $n = 4$) of the injected [^{3}H]Ch18:2-OH label was already associated with the liver, as compared with 2.4±0.25% (S.E.M.; $n = 4$) for native Ch18:2. At 30 min after injection of [^{3}H]Ch18:2-OH-labelled HDL, 27.0±2.0% (S.E.M.; $n = 4$) of the injected dose of label was taken up by the liver, as compared with 11.6±0.8% (S.E.M.; $n = 4$) after injection of [^{3}H]Ch18:2. At 30 min after injection, 39.1±3.3% (S.E.M.; $n = 4$) of [^{3}H]Ch18:2-OH and 64.0±7.3% (S.E.M.; $n = 4$) of [^{3}H]Ch18:2 were still present in the serum. The main difference in clearance rate and liver uptake of both labels occurred during the first 5 min after injection. Because the serum decay of [^{3}H]Ch18:2-OH and [^{3}H]Ch18:2 could not be accounted for by liver uptake, we also analysed the distribution of label in extrahepatic tissues.

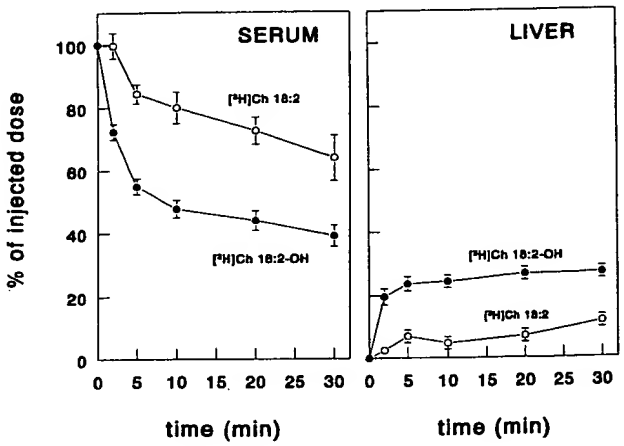


Figure 1 Serum decay and liver association of HDL-[^{3}H]Ch18:2-OH and HDL-[^{3}H]Ch18:2 in the rat

300–400 μg (500000 d.p.m.) of [^{3}H] cholesteryl ester-labelled HDL {[^{3}H]Ch18:2-OH (●) or [^{3}H]Ch18:2 (○)} were injected into the inferior vena cava of anaesthetized rats. At indicated time points serum was withdrawn from the inferior vena cava and a liver lobule was tied off, weighed, combusted in a Hewlett-Packard sample oxidizer 306 and counted for radioactivity. Serum decay and liver association were calculated. A correction was made for the contribution of serum to the total liver-associated radioactivity. Data are expressed as a percentage of injected dose (±S.E.M.) of four experiments.

Tissue distribution

Tissue distribution studies at 30 min after injection of [^{3}H]Ch18:2-OH- and [^{3}H]Ch18:2-labelled HDL indicate that the liver is the major site of uptake for both CE (Figure 2). The skin, muscles and adrenals are the other main uptake sites. Interestingly, of all organs analysed, only the adrenals, in addition to the liver, showed a preferential uptake of [^{3}H]Ch18:2-OH from HDL as compared with [^{3}H]Ch18:2. The adrenals contained 5.1±0.6% and 2.0±0.7% (S.E.M.; $n = 4$) of the injected dose of [^{3}H]Ch18:2-OH and [^{3}H]Ch18:2 respectively.

Intrahepatic cellular distribution

To identify the intrahepatic uptake sites for [^{3}H]Ch18:2-OH and [^{3}H]Ch18:2 from HDL, the various liver cell types, i.e. parenchymal, Kupffer and liver endothelial cells, were isolated at 10 min after injection. The experiments shown in Table 1 were performed with ^{125}I-labelled HDL (to measure particle uptake) and HDL labelled with either [^{3}H]Ch18:2-OH or [^{3}H]Ch18:2. For total liver, the association of [^{3}H]Ch18:2OH-HDL and [^{3}H]Ch18:2-HDL were respectively 15- and 8-fold higher as

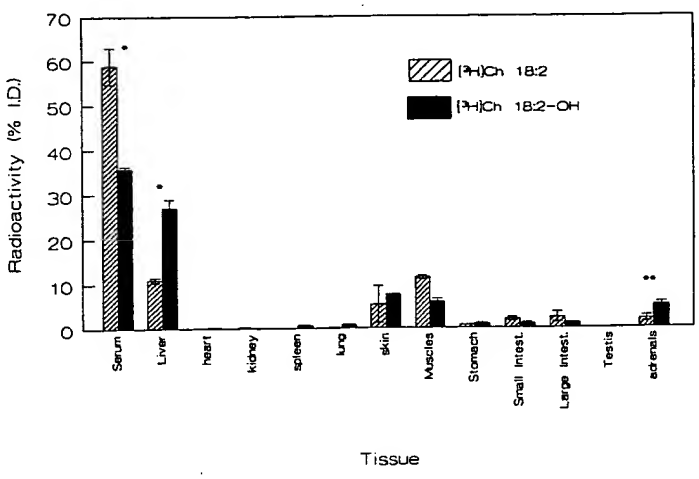


Figure 2 Tissue distribution of HDL-[^{3}H]Ch18:2-OH and HDL-[^{3}H]Ch18:2 at 30 min after injection in the rat

[^{3}H]CE-labelled-HDL (300–400 μg; 500000 d.p.m.) ([^{3}H]Ch18:2-OH, shaded bars or [^{3}H]Ch18:2, hatched bars) were injected into the inferior vena cava of anaesthetized rats. At 30 min after injection the amount of radioactivity in tissue samples, as a percentage of the injected dose, was determined after combustion in a Hewlett-Packard sample oxidizer 306 and counting for radioactivity. A correction was made for the contribution of serum to the total tissue-associated radioactivity. The total recovery of label was 94% and 86% of the injected radioactivity for [^{3}H]Ch18:2 and [^{3}H]Ch18:2-OH respectively. Values are means ± S.E.M. of four experiments. * Indicates significant difference between [^{3}H]Ch18:2-OH and [^{3}H]Ch18:2; $P < 0.005$. ** Indicates significant difference between [^{3}H]Ch18:2-OH and [^{3}H]Ch18:2, $P < 0.05$ (unpaired Student's *t*-test).

Table 1 *In vivo* distribution of HDL-[^{3}H]Ch18:2-OH, HDL-[^{3}H]Ch18:2 and ^{125}I-labelled HDL between parenchymal, liver endothelial and Kupffer cells, at 10 min after injection

At 10 min after injection of [^{3}H]cholesteryl ester, [^{3}H]Ch18:2-OH or [^{3}H]Ch18:2 labelled HDL or ^{125}I-labelled HDL, the liver was perfused and parenchymal cells (PC), endothelial cells (EC) and Kupffer cells (KC) were isolated at 4 °C. Values, expressed as a percentage of ID × 10^3/mg of cell protein, represent the means of two experiments ± the variation of the individual experiments; ID, injected dose.

	Radioactivity [10^3 × ID/mg of cell protein (%)]		
Cell fraction	^{125}I	[^{3}H]Ch18:2	[^{3}H]Ch18:2-OH
Total liver cells	0.75 ± 0.50	9.8 ± 2.0	14.7 ± 1.6
PC	0.27 ± 0.05	4.7 ± 0.6	14.6 ± 4.0
EC	1.5 ± 0.0	2.5 ± 0.3	2.2 ± 0.2
KC	1.8 ± 0.1	1.0 ± 0.7	1.1 ± 0.2

compared with ^{125}I-labelled HDL. Both types of CE from HDL are taken up by parenchymal cells to a much higher extent than the apolipoprotein moiety, which is indicative of selective delivery. *In vivo* HDL-associated cholesteryl ester hydro(pero)xide uptake by parenchymal cells was 3.2-fold higher for [^{3}H]Ch18:2-OH as compared with [^{3}H]Ch18:2 uptake. For liver endothelial cells and Kupffer cells, in agreement with earlier data [10], no selective uptake of native CE was observed. For the non-parenchymal cells, the uptake of [^{3}H]Ch18:2-OH from HDL did not exceed the uptake of [^{3}H]Ch18:2, and consequently, the contribution of liver endothelial cells and Kupffer cells to the total liver uptake of both CE is very low. Taking into account the predominance of parenchymal cell protein in liver, it can be calculated that 99 ± 0.2% and 97 ± 0.1% of [^{3}H]Ch18:2-OH and [^{3}H]Ch18:2 respectively were taken up by the liver parenchymal cells. The higher liver uptake of [^{3}H]Ch18:2-OH can therefore be solely accounted for by the contribution of the liver parenchymal cells.

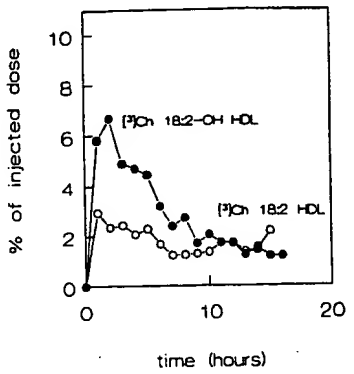
Biliary secretion of [^{3}H]-radioactivity

In a previous study [10] it was shown that CE from HDL, which were selectively delivered to liver parenchymal cells, were efficiently secreted into bile after conversion into bile acids. In order to assess whether the increased parenchymal cell uptake of HDL-associated [^{3}H]Ch18:2-OH is also coupled to an efficient secretion route into bile, rats were equipped with permanent catheters in the bile duct, duodenum and the vena jugularis. The kinetics of appearance of biliary radioactivity was analysed up to 15 h after injection of [^{3}H]Ch18:2-OH- and [^{3}H]Ch18:2-labelled HDL into the vena jugularis. After injection of [^{3}H]Ch18:2-OH-labelled HDL, radioactivity appeared rapidly in bile. Within 1 h after injection, 5.8% of the injected dose was already secreted into bile as compared with 2.9% when [^{3}H]Ch18:2-labelled HDL was injected (Figure 3, top). During the first 10 h, biliary secretion of radioactivity from [^{3}H]Ch18:2-OH-labelled HDL remained higher as compared with [^{3}H]Ch18:2. Within this period, 41% of the injected dose of [^{3}H]Ch18:2-OH had been secreted into bile, while 16% of the radioactivity from [^{3}H]Ch18:2 was secreted (Figure 3, bottom). Bile analysis revealed that for both [^{3}H]Ch18:2-OH and [^{3}H]Ch18:2, the radioactivity in the bile was mainly present in the form of bile acids (99% versus 96% respectively).

In addition to biliary secretion, the liver is also able to return surplus CE to the circulation via secretion of newly synthesized VLDL. In order to assess whether this pathway significantly contributes to the turnover of HDL-associated CE, blood samples were taken at different time points (up to 4 h) and serum was subjected to density gradient ultracentrifugation. Analysis of the density gradient fractions revealed, however, that less than 5% of the injected dose was present in lipoprotein fractions other than HDL (results not shown).

In vitro incubations with freshly isolated liver parenchymal cells

Liver cell distribution studies clearly demonstrated that the liver parenchymal cells were exclusively responsible for the preferential



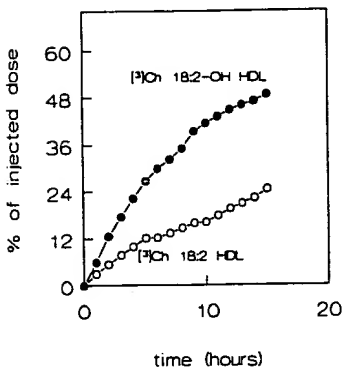


Figure 3 Biliary secretion of [^{3}H]-radioactivity expressed as a percentage of the injected dose per hour: (top) and as cumulative percentage of the injected dose; and (bottom) after administration of HDL-[^{3}H]Ch18:2-OH or HDL-[^{3}H]Ch18:2 in rats

Bile was collected for 15 h in 1 h time intervals after injection of 300–400 μg of HDL (500000 d.p.m.) {[^{3}H]Ch18:2-OH (●) or [^{3}H]Ch18:2 (○)} in unrestrained catheterized rats. Values, expressed as a percentage of injected dose (top) or as cumulative percentage of the injected dose (bottom) represent the means of two experiments.

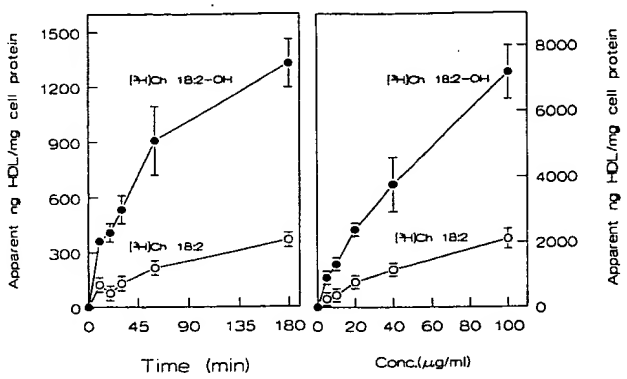


Figure 4 Relation of the incubation time (left) and the concentration of HDL-[^{3}H]Ch18:2-OH or HDL-[^{3}H]Ch18:2 (right) to the association of HDL-[^{3}H]Ch18:2-OH or HDL-[^{3}H]Ch18:2 with rat hepatocytes

Left: cells were incubated with 10 μg/ml of labelled HDL-[^{3}H]Ch18:2-OH (●) or [^{3}H]Ch18:2 (○) for the indicated amount of time at 37 °C in DMEM with 2% (w/v) BSA. The values are corrected for non-specific cell association in the presence of a 20-fold excess of HDL. The results are given as means ± S. E. M. ($n = 3$). Right: cells were incubated for 3 h at 37 °C with the indicated concentration of labelled HDL-[^{3}H]Ch18:2-OH (●) or HDL-[^{3}H]Ch18:2 (○) in DMEM with 2% (w/v) BSA. The values shown here are corrected for the non-specific cell association in the presence of a 20-fold excess of HDL. The results are given as means ± S.E.M. of three experiments.

liver uptake of [^{3}H]Ch18:2-OH over [^{3}H]Ch18:2 from HDL. The kinetics of the selective uptake process of [^{3}H]Ch18:2-OH and [^{3}H]Ch18:2 from HDL were studied further *in vitro* with freshly isolated liver parenchymal cells (Figure 4). Data are expressed in terms of apparent particle uptake as suggested by Pittman et al. [8], i.e. the amount of apparent HDL protein taken up is calculated from the amount of CE tracer associated with cells. Figure 4 (left) shows the time-dependent uptake of [^{3}H]Ch18:2-OH or [^{3}H]Ch18:2 by parenchymal cells. In agreement with the results obtained during serum decay and liver uptake studies, uptake of [^{3}H]Ch18:2-OH was characterized by a very rapid initial transfer of the oxidized label to the parenchymal cells. Within 3 min, the amount of cell-associated [^{3}H]Ch18:2-OH was almost similar to the uptake of [^{3}H]Ch18:2 during the entire 180 min incubation. At the end of the incubation, the amount of cell-associated [^{3}H]Ch18:2-OH exceeded [^{3}H]Ch18:2 uptake 4-fold.

We also compared the concentration-dependent uptake of [^{3}H]Ch18:2-OH-HDL and [^{3}H]Ch18:2-HDL by parenchymal cells (Figure 4, right). From the results shown in Figure 4 (right) it is evident that [^{3}H]Ch18:2-OH uptake exceeds [^{3}H]Ch18:2 uptake. The affinity of cell association was only slightly higher for CE than for [^{3}H]Ch18:2-OH (half-maximal association values were 109.9 μg/ml and 93.7 μg/ml for [^{3}H]Ch18:2-OH and [^{3}H]Ch18:2 respectively). However, the maximal cellular association after 3 h was 3.6-fold higher for [^{3}H]Ch18:2-OH as compared with [^{3}H]Ch18:2 association.

DISCUSSION

The generally accepted anti-atherogenic effect of HDL may be based upon several mechanisms. The protective role of HDL is classically associated with reverse cholesterol transport, as first proposed by Glomset [5]. This model describes the uptake and esterification of excess cholesterol from macrophages and other cell types, and the subsequent transport and delivery of CE to the liver. HDL is considered to play a major role in this process, especially during the initial cholesterol uptake from cells [7,33,34]. In addition to this generally accepted concept, HDL may also protect against oxidation of LDL, which is suggested to play an important role in the development of atherosclerosis.

HDL is suggested to accept oxidized lipids from LDL as part of an anti-oxidative process [15–18]. The first step in LDL oxidation involves hydroperoxide formation [2]. Because HDL is the predominant carrier of CEOOH in humans [17], it was expected that transfer from LDL to HDL could occur. Indeed Christison et al. [18] have shown recently that CETP-mediated transfer of CEOOH is possible. HDL-associated CEOOH are rapidly reduced to CEOH [16]. Our present data indicate that CEOH from HDL are more rapidly removed from the blood circulation *in vivo* than normal HDL-associated CE. Liver uptake is mainly responsible for this rapid removal, although within the liver only parenchymal cells exert this property. The uptake by parenchymal cells is coupled to efficient biliary excretion. Among the other organs tested, only the adrenals showed increased CEOH uptake as compared with CE.

In general, clearance of HDL-CE from the blood circulation is caused mainly by the liver and steroid-forming tissues [8–10]. The kinetics of liver uptake of CEOH from HDL seems to differ from native CE uptake only in the initial uptake phase, after which the liver uptake increases only slowly, at an equal rate to native HDL-associated CE. The liver uptake profile of native Ch18:2 is in accordance with previously published data [35]. The uptake of both native and oxidized CE from HDL by the liver is characterized by a preferential uptake of the CE as compared with the protein moiety, and in the liver this is coupled to efficient biliary secretion. Also, the steroid-synthesizing organs utilize this selective uptake mechanism for CE [8]. Within the liver, only the parenchymal cells are able to perform this process [10]. Interestingly, only the parenchymal cells show increased CEOH uptake as compared with native CE uptake.

The exact mechanism of selective uptake of native CE by liver parenchymal cells is still largely unknown. Rinninger et al. [36] showed that there are distinct sites on liver parenchymal cell-membranes for binding of the protein moiety of HDL and CE uptake. Inhibition of the protein-binding site did not result in changes in the uptake of CE. Work by Rothblat's group [37,38] indicates that transfer of CE between HDL and cells might be dependent on lipid interactions between HDL and the plasma membrane rather than on receptor-mediated binding to cells. It can be anticipated that the increased decay and liver uptake of CEOH, as compared with native CE, is caused by the change in physical attributes of the oxidized CE. The introduction of hydroxyl groups yields better solubility in water, enabling the CEOH to transfer more efficiently between HDL and cellular membranes. However, based upon our experiments *in vivo*, it can be concluded that this does not lead to increased uptake by all cell types, but apparently only liver parenchymal cells and the adrenals are able to utilize the greater water solubility for increased uptake. Interestingly, muscles seem to prefer native CE uptake as compared with CEOH.

Analysis of the kinetics of radioactive bile-acid secretion from administered labelled native and oxidized CE, indicate that the increased uptake of CEOH from HDL by parenchymal cells is coupled to an increased rate of biliary secretion, indicating that the efficient liver uptake might indeed lead to the irreversible removal of CEOH from the body. The association of enzymic activities with HDL, together with efficient clearance route of HDL-associated oxidized CE, as exerted by liver parenchymal cells, may work synergistically to detoxify lipid hydroperoxides and thereby protect LDL from oxidation *in vivo*.

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